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Trehalose Toxicity in *Cuscuta reflexa*[1]

CELL WALL SYNTHESIS IS INHIBITED UPON TREHALOSE FEEDING

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ABSTRACT

α,α-Trehalose induced a rapid blackening of the terminal 2.5-centimeter region of excised *Cuscuta reflexa* Roxb. vine. The incorporation of radioactivity from [^{14}C]glucose into alkali-insoluble fraction of shoot tip was markedly inhibited by 12 hours of trehalose feeding to an excised vine. This inhibition was confined to the apical segment of the vine in which cell elongation occurred. The rate of blackening of shoot tip explants was hastened by the addition of gibberellic acid A_3, which promoted elongation growth of isolated *Cuscuta* shoot tips. The symptom of trehalose toxicity was duplicated by 2-deoxyglucose, which has been shown to be a potent inhibitor of cell wall synthesis in yeast. The observations suggest that trehalose interferes with the synthesis of cell wall polysaccharides, the chief component of which was presumed to be cellulose.

The nonreducing diglucoside, α,α-trehalose, has not been found in the angiosperms, although constitutive trehalose-hydrolyzing activity (trehalase, EC 3.2.1.28) has been found in all plants examined (4–6, 14). The ability of plant tissues to utilize trehalose for growth *in vitro* has been demonstrated (6, 14). We observed a toxic effect of trehalose in an angiospermic plant, *Cuscuta reflexa* (dodder). This plant had an exceptionally low trehalase activity; as a result, trehalose accumulated in shoot tip cultured *in vitro* and induced its rapid blackening (14). A striking feature of trehalose toxicity in a cut vine was that blackening occurred only in the region of cell elongation although trehalose accumulated throughout the length of the vine (14). The toxicity symptom in shoot tips was delayed by the addition of a growth promotory sugar to the culture medium (15). These observations suggested that trehalose interferes in a distinctive metabolic process in the growing region of the vine which primarily involves the metabolic pathway of sugars. In this paper we present observations which suggest that trehalose may interfere with the synthesis of cell wall material.

MATERIALS AND METHODS

Culture Method. The shoot tips of *C. reflexa* Roxb. were cultured as described by Maheshwari *et al.* (10). The culture medium lacking sugar is designated as BM.[3]

Incorporation of [^{14}C]Glucose. Vines (8 cm) were marked with ink at 2 cm below the apex and placed with their cut ends dipped in BM or BM + 2% trehalose. At the end of growth period, shoot tips from three identically treated vines were excised at the mark and placed upright in a test tube containing 0.1 ml BM + [U-^{14}C] glucose at 30°C. After the incubation period, the shoot tips were used for analysis. The difference between the initial and the final radioactivity in the medium was taken as the uptake of [^{14}C] glucose.

Tissue Fractionation. Shoot tips were extracted three times with 10 ml portions of hot 80% ethanol (v/v). The residue was homogenized in 80% ethanol, extracted at 80°C for 10 min, centrifuged and the extraction was repeated. All ethanol-extracts were combined. The alcohol-insoluble residue was fractionated by the method of Brett (2) to obtain alkali-soluble and alkali-insoluble fractions.

The combined ethanol-extract was evaporated to dryness under reduced pressure between 40 and 45°C. The residue was dissolved in 0.4 ml water, mixed with 1 ml methanol + 0.5 ml chloroform and transferred to a centrifuge tube. To this, 0.5 ml chloroform and 0.5 ml water were added, vortexed and centrifuged. The lower chloroform phase was considered as the lipid fraction. The upper aqueous fraction was consecutively passed through small columns of Dowex 50 (H^+ form) and Dowex 1 ($HCOO^-$ form). The columns were washed with 20 ml of deionized H_2O and the eluate was designated as the neutral fraction. The basic fraction was eluted from the Dowex 50 column with 20 ml of 2 N NH_4OH, whereas the acidic fraction was eluted from the Dowex 1 column with 20 ml 6 N HCOOH. NH_4OH from the basic fraction and HCOOH from the acidic fraction were removed over H_2SO_4 and NaOH, respectively, in a vacuum desiccator. The neutral, acidic, and basic fractions were evaporated to dryness and dissolved in water.

Solutions for radioactivity measurements were spotted on 1.8 × 1.8 cm Whatman No. 3 paper, dried, and counted in a toluene-based scintillation fluid containing methanol (13).

Chemicals. All biochemicals used were bought from Sigma. [U-^{14}C]Glucose (114 mCi/mmol) was bought from Bhabha Atomic Research Centre, Trombay, India. [U-^{14}C]Trehalose (540 mCi/mmol) was bought from Radiochemical Centre, Amersham, England.

RESULTS

Incorporation of [^{14}C]Glucose. Radioactivity from [^{14}C]glucose has been shown to be incorporated into plant cell wall polysaccharides (3) and it was therefore chosen for the present study. Incorporation of label was studied at intervals of 12 h up to 36 h because, until this time, difference between treated and control shoot tips was not visible. The uptake of label in trehalose-treated explants was affected to a small extent (about 8%). Radioactivity in acidic, basic, lipid, and alkali-soluble fractions was about comparable in control and trehalose-treated tissue (data not shown). Radioactivity in the neutral and alkali-insoluble fractions of the trehalose-treated tissues was respectively 40% and 20% less

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[3] Abbreviation: BM, basal medium.

Table I. *Effect of Trehalose Feeding on the Incorporation of [^{14}C]Glucose into Cell Wall Fractions of Cuscuta reflexa Shoot Tips*

Excised vines (8 cm) were marked with ink 2 cm below the apex and placed with cut ends dipped in either BM (control) or BM + 2% trehalose (treated) for 24 h. Shoot tips from three identically treated vines were cut at the mark and fed 8.4 × 10^6 cpm of [^{14}C]glucose for 105 min and combined for analysis.

Experiment	Treatment	Uptake	Radioactivity	
			Alkali-insoluble fraction	Alkali-soluble fraction
			cpm × 10^{-2}	
1	Control	9382	606 (6.5)[a]	359 (3.5)
	Treated	8486	263 (3.1)	256 (3.0)
2	Control	7793	544 (7.0)	379 (4.8)
	Treated	8448	290 (3.4)	228 (3.4)

[a] Numbers in parentheses, radioactivity expressed as percent of uptake.

Table II. *Effect of Trehalose Treatment on the Incorporation of [^{14}C] Glucose into Cell Wall Fractions of Apical and Subapical Regions of Cuscuta Vine*

Apical (0–2.5 cm) and subapical (2.5–5 cm) regions were marked in cut vines (8 cm). The vines were placed with cut ends dipped in either BM (control) or BM + 2% trehalose (treated) for 24 h. Corresponding segments from three identically treated vines were cut and fed [^{14}C]glucose (2 × 10^6 cpm) for 2 h and then combined for analysis.

Region of Vine	Treatment	Uptake	Radioactivity	
			Alkali-insoluble fraction	Alkali-soluble fraction
			cpm × 10^{-2}	
Apical	Control	2054	123 (6.0)[a]	59 (2.9)
	Treated	3062	58 (1.9)	43 (1.4)
Subapical	Control	6604	105 (1.6)	48 (0.7)
	Treated	5343	93 (1.7)	64 (1.2)

[a] Numbers in parentheses, radioactivity expressed as percent of uptake.

than in the control tissue. As reported before (15), the low incorporation of radioactivity in the neutral fraction of trehalose-treated shoot tip was associated with the inhibition of sucrose synthesis.

A study of incorporation into the alkali-insoluble fraction (Table I) showed that up to 50% lower incorporation of radioactivity from [^{14}C]glucose into this fraction occurred consequent to trehalose feeding. The incorporation into the alkali-soluble fraction was affected to a small extent. The lower incorporation of radioactivity in the alkali-insoluble fraction of trehalose-treated shoot tip suggested that cell wall polysaccharide synthesis was affected.

Because trehalose toxicity was confined to the terminal region of *Cuscuta* vine, we compared the incorporation of [^{14}C]glucose into cell wall material in the apical and the subapical regions of shoot tip. The results of this experiment (Table II) showed that the incorporation of label into cell wall material in the apical region (0–2.5 cm) was less than in the subapical region (2.5–5 cm). The apical region of the vine was the zone of maximal elongation growth (14). These observations support the idea that trehalose inhibits cell wall synthesis—a major process during the elongation of plant cells.

Incorporation of [^{14}C]Trehalose. Shoot tip explants, (2.5 cm) were cultured for 60 h in medium containing 1% trehalose and 1.24 μCi [^{14}C]trehalose, following which the tissue was fractionated for the distribution of radioactivity. The radioactivity in each fraction, expressed as percentage of total radioactivity in all fractions, was as follows: alkali-insoluble: 15; alkali-soluble: 12.5; lipid: 19.7; acidic: 15.2; basic: 6; and neutral: 31.6. The results showed that trehalose was metabolized in the tissue.

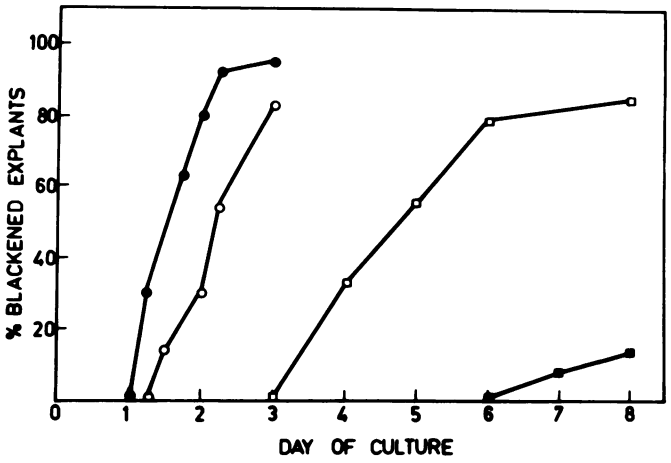

FIG. 1. Time course of trehalose-induced blackening of shoot tips of *Cuscuta* cultured *in vitro* in the absence and in the presence of GA_3 14.4 μM). Shoot tips (2.5 cm) were cultured in BM + 2% trehalose (○, without GA_3; ●, with GA_3) or in BM + 2% trehalose + 2% sucrose (■, without GA_3; □, with GA_3). Number of shoot tips cultured in each treatment was 20 to 25.

Effect of Gibberellic Acid A_3. If trehalose inhibits cell wall polysaccharide synthesis, then a treatment which activates cell (wall) growth may be expected to hasten the onset of toxicity. We have previously reported that GA_3 markedly promotes cell enlargement in excised shoot tips of *Cuscuta* (10). Therefore, we studied the effect of GA_3 on trehalose toxicity. As shown in Figure 1, the rate of trehalose-induced blackening was faster in shoot tips cultured in the presence of this hormone. For instance, at 48 h only 30% of the control (no GA_3) had blackened, whereas about 80% of the explants blackened in the presence of GA_3. As reported before (14), the addition of sucrose to the culture medium resulted in the delaying of blackening. Therefore, the effect of GA_3 addition when both trehalose and sucrose were fed to the tissue was studied (Fig. 1). On day 6, about 80% of the explants had blackened when GA_3 was present in the medium but none when the hormone was absent.

GA_3 could hasten the toxic effect of trehalose by promoting the uptake of trehalose or by activating the utilization of endogenous sugars and thereby altering the ratio of trehalose to soluble sugars within the tissue. To obtain information on these possibilities, the shoot tips cultured for 108 h in media containing 2% trehalose + 5% sucrose in the absence and presence of the hormone were analyzed as before (15). However, the contents of total sugars, reducing sugars, and the amount of trehalose accumulated were not significantly different (data not shown) and the possibilities mentioned above were discounted. Rather, it seemed likely that GA_3 activated cell wall loosening and opened up sites for trehalose action.

Effect of 2-Deoxyglucose. The results of the experiments described above, suggesting that trehalose may inhibit cell wall synthesis, led us to examine the effect of 2-deoxyglucose which has been shown to inhibit cell wall formation in yeast (1, 7). The effect of 2-deoxyglucose on *Cuscuta* was studied as for trehalose (14). 2-Deoxyglucose induced blackening and death of shoot tip explants at concentrations as low as 0.02% in 3 to 4 days. The toxicity syndrome was identical to that caused by trehalose (14).

DISCUSSION

The reduced incorporation of radioactivity from [^{14}C]glucose into the alkali-insoluble fraction suggests that biosynthesis of cell wall polysaccharides is affected in trehalose-fed shoot tips of *Cuscuta*. We are, therefore, inclined to the view that the site of toxicity is the cell wall. This view conforms with the most characteristic feature of the toxicity syndrome, *i.e.* the confinement of blackening to the actively growing region of the vine (14). Moreover, this idea is supported by the observation that sugars which promote elongation growth of excised *Cuscuta* shoot tips antagonize the toxic effect of trehalose by delaying the onset of blackening (14). Trehalose apparently competes with other sugars for utilization in a pathway which is a distinctive feature of the growing cell, namely, cell wall synthesis. For example, sucrose has been postulated to serve as a source of UDP-glucose required in biosynthesis of cell wall polysaccharides (11, 12). Furthermore, the idea that the cell wall is the site of toxicity is sustained by the observation that under conditions of hormonal activation of cell (wall) growth, the onset of toxicity is markedly hastened.

The alkali-insoluble fraction is generally considered to be celluose (2). The toxicity in *Cuscuta* may result if trehalose interferes with the process of addition of new glucose units into glucan at the time of cell wall loosening (9) during elongation growth. Under these conditions, cell wall loosening would occur unaccompanied by cell wall synthesis. This would explain the softening of tissue and oozing of fluid in the growing region of the *Cuscuta* vine (14). A similar mechanism of lysis of yeast cells by 2-deoxyglucose has been proposed (7). It is noteworthy that besides trehalose and its analogs (14), only 2-deoxyglucose was found to induce blackening of shoot tip of *Cuscuta*.

The possibility of introduction of trehalose as a unit into cellulose and thus causing abrupt termination of chain growth was investigated by studying the incorporation of [^{14}C]trehalose in *Cuscuta* shoot tips. However, the heterogeneous distribution of radioactivity showed that [^{14}C]trehalose was, at least partially, hydrolyzed in the tissue resulting in the labeling of different cellular fractions. We reported earlier (14) that extracts of *Cuscuta* have trehalose-hydrolyzing activity, though low. Therefore, from [^{14}C]trehalose feeding experiments it could not be ascertained whether trehalose as a unit was introduced into cellulosic material or not. Inasmuch as trehalose toxicity symptom was reproduced by trehalosamine (14), an analog of trehalose and an inhibitor of trehalase (8), the availability of radiolabeled trehalosamine may be useful in determining its introduction into glucan molecules.

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